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Ligand interaction binding site and G protein activation of the mu opioid receptor

Q1 Xu Cui [a,b], Alexei Yeliseev [c], Renyu Liu [b,*]

Q2 [a] Department of Anesthesiology, Beijing Tongren Hospital, Capital Medical University, Beijing, China
[b] Department of Anesthesiology and Critical Care, Perelman School of Medicine at the University of Pennsylvania, USA
[c] National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Department of Anesthesiology and Critical Care, Hospital of University of Pennsylvania, USA

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ABSTRACT

With the recently solved crystal structure of the murine mu opioid receptor, the elucidation of the structure function relationships of the human mu receptor becomes feasible. In this study, we analyzed the available structural information along with ligand binding and G protein activation of human mu receptor. Affinity determinations were performed in a HEK293 cell line stably transfected with the human mu opioid receptor for 6 different agonists (morphine, DMAGO, and herkinorn) and antagonists (naloxone, beta-Funaltrexamine, and Norbinaltorphimine) based on the method. G protein activation was investigated in membrane preparations containing human mu receptors treated with the agonist, partial agonist, or antagonist compounds. 4DKL.pdb was utilized for structural analysis and docking calculations for 28 mu receptor ligands. The predicted affinities from docking were compared with those experimentally determined. While all known ligands bind to the receptor through the same binding site that is large enough to accommodate molecules of various sizes, interaction with D147 (D149 in human mu receptor) is essential for binding. No distinguishable interaction pattern in the binding site for agonist, partial agonist, or antagonist to predict pharmacological activities was found. The failure to reconcile the predicted affinities from docking with experimental values indicates that the receptor might undergo significant conformational changes from one state to the other states upon different ligand binding. A simplified model to understand the complicated system is proposed and further study on these multiple conformations using high resolution structural approaches is suggested.

1. Introduction

The therapeutic use and abuse of opioids has soared globally in recent years (Devi, 2011; Manchikanti and Singh, 2008; Kuehn, 2007a,b, 2009; Manchikanti, 2006). Between 1999 and 2002, the number of fatal opioid analgesic poisonings has increased by 91% while methadone-related deaths from 1999 to 2004 have increased by 390% (Paulozzi et al., 2006a, 2006b). The White House Budget Office estimates that, in addition to the human toll, opioid abuse may contribute up to $300 billion per year in direct healthcare costs (White et al., 2005). The pharmacological targets of opioids are opioid receptors which currently include 4 family members: mu, delta, kappa and nociception receptors (Cox, 2012). Each receptor type is responsible for different pharmacological effects and receptor-specific functional outcome despite of the high sequence homology among these receptors (67% to 76%) (Cox, 2012). The mu opioid receptor mediates pain perception and is responsible for the above mentioned problem related to opioid usage (addiction, respiratory depression and other side effects).

Crystal structures of these four receptors have been recently reported (Granier et al., 2012; Manglik et al., 2012; Thompson et al., 2012; Wu et al., 2012). Current research takes advantage of the solved crystal structure of the transmembrane portion of an engineered murine mu receptor—which displays high homology with its human counterpart (Manglik et al., 2012). The characterization of the structure of the mu opioid receptor should be part of the solution for the problems noted above as such information would further elucidate the mechanisms of these receptors. Furthermore, it would undoubtedly aid in the design and/or discovery of novel and potentially safer medication by extending structural insights and analyzing docking of other ligand in crystallized structure (Jacobson and Costanzi, 2012).

In particular, information from this crystal structure (Manglik et al., 2012) can be potentially exploited to further elucidate the structure–function relationship of the human mu receptor and thus advance our understanding of mechanisms mediating pain, addiction, and respiratory depression. Many important functional properties of the human mu opioid receptor—such as its ability to bind with various agonists, partial agonists, and antagonists, and its capacity to activate cognate guanine nucleotide-binding,

* Correspondence to: Department of Anesthesiology and Critical Care, University of Pennsylvania School of Medicine, 336 John Morgan building, 3620 Hamilton Walk Philadelphia, PA 19104, USA. Tel.: +1 2156623750; fax: +1 2153495078.
E-mail address: liur@uphs.upenn.edu (R. Liu).

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G proteins, upon agonist binding—can be analyzed in a laboratory setting. G protein activation is required in most of the cases for opioid receptors and other GPCRs to trigger pharmacological events in the living organisms (Cox, 2012). It is critical to relate the receptor structure information to these downstream pharmacological effects by using a variety of modern biochemical and biophysical techniques since the co-crystallization of the receptors with G proteins and X-ray analysis remains technically very challenging.

Given the high homology between the murine and human mu opioid receptors, we investigated whether the information from the newly available crystal structure could provide insights into the biological functions of the human receptor, especially in regard to ligand binding and in vitro G protein activation.

2. Materials and methods

Membrane preparations of recombinant human mu opioid receptor expressed in the mammalian cell line Chem-5 and used for G protein activation studies were obtained from Millipore (Billerica, MA, USA). All opioid ligands were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were reagent grade or higher. Herkinorin was purchased from Ascent Scientific LLC (Princeton, NJ, USA). All chemicals were used without further purification.

Although the crystal structure of the human mu opioid receptor is not available, a sequence analysis of the human (uniprot accession number P35372, http://www.uniprot.org/) and mouse (uniprot accession number P42866) μ opioid receptors shows a sequence identity of 94% for the entire sequence. The similarity of the sequences in the region solved in the crystal structure (PDB access code: 4DKL (Manglik et al., 2012)) is 99%. Since differences between these sequences occur outside of the binding pocket, results from binding pocket analysis and docking experiments will be equally relevant for human mu opioid receptor.

2.1. Binding pocket volume and area determination

The binding pocket volume and area information was analyzed using CASTp (http://sts.bioengr.uic.edu/castp/calculation.php), an online binding pocket analysis tool (Liang et al., 1998). The default value of 1.4 Å was used for calculation. The binding pocket image was generated using PyMOL (Version 1.3, Schrödinger, LLC.; http://www.pymol.org/) along with a CASTp PyMOL plugin (CASTpyMOL v2.0, http://sts.bioengr.uic.edu/castp/pymol.php).

2.2. Docking calculations

Docking calculations for the structure of the murine mu receptor (PDB access code: 4DKL (Manglik et al., 2012)) were carried out using DockingServer (http://www.dockingserver.com) (Bikadi and Hazai, 2009) as previously described (Liu et al., 2012). Semi-empirical charges calculated by MOPAC2009 were added to the ligand atoms (http://openmopac.net/MOPAC2009.html) (Stewart, 1990). Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added to the receptor using AutoDock tools provided by the server. Grid maps of $30 \times 30 \times 30$ Å^3 grid points with 0.375 Å spacing centered at the known ligand binding site were generated using the Autogrid program (Morris et al., 1996, 2009). Opioid agonist, partial agonist, and antagonist searches were performed using the Solis and Wets local search method with a Lamarckian genetic algorithm (Solis and Wets, 1981). Initial position, orientation, and torsions of the ligand molecules were set randomly. The predicted site with a dominant energy was chosen for subsequent analysis. The estimated binding constant (Ki) was derived from the equation $\Delta G = -RTlnK$, where ΔG is directly calculated during docking runs using the Autodock scoring function.

A total of 26 ligands for the opioid receptor—which included full agonists, partial agonists and antagonists—were selected for docking calculations based on affinities experimentally obtained in this study, by using the same methodology for affinity determination from a study published recently (Volpe et al., 2011) (see Table 1). The three-dimensional coordinates of the tested opioids were obtained from the PubChem database (http://pubchem.ncbi.nlm.nih.gov/). The residues interacting with the ligands were analyzed in an attempt to find potential patterns for ligand binding. PyMOL was used to render the graphics for presentation.

2.3. Affinity determinations and correlation analysis

Affinity determinations for agonists (morphine, DAMGO, and herkinorn) and antagonists (naloxone, beta-Funaltrexamine, and Norbinaltorphimine) were performed in a HEK293 cell line stably transfected with the human mu opioid receptor as previously described (Roth et al., 1981). Although most of the affinity data are available in literature, we chose some of the typical agonists from different categories (morphine, small peptide, non-opioid mu opioid receptor) and antagonists to determine the affinity using the same methodology to avoid technical variances. The determined affinities are compared with those from the docking prediction. The affinities available for 16 full agonists or partial agonists from the same study published recently (Volpe et al., 2011) were compared with those predicted by docking. Potential correlations were determined using GraphPad Prism (V5.04, GraphPad Software, La Jolla, CA).

2.4. G protein activation by receptor treated with agonists, partial agonists and antagonists

The effects of specific mu opioid receptor ligands on the activation of the recombinant receptor expressed in mammalian cell membranes were investigated by measuring G protein activation in vitro. The full agonist DAMGO ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin), partial agonist nalbuphine, and antagonist naloxone were utilized.

The assay reports the initial rates of activation of heterotrimeric G proteins ($G\alpha i_{i1}\beta_1\gamma_2$) on an agonist-bound receptor by measuring the accumulation of [^{35}S]–GTPγS (non-hydrolyzable analog of GTP) bound to the activated $G\alpha_{i1}$ subunit. Myristoylated $G\alpha_{i1}$ was expressed in *E. coli* and purified as previously described (Mumby and Linder, 1994). Recombinant human $\beta_1\gamma_2$ subunits of G protein were expressed in baculovirus-infected Sf9 cells and purified as previously described (Wildman et al., 1993). The G protein activation assay was conducted as follows (final concentrations in 50 μl reaction mixture are given in parentheses): the membrane sample was diluted into ice-cold 10 mM MOPS buffer to reach a protein concentration of 40 ng/μl. 10 μl of the diluted dispersion were dispensed into pre-siliconized glass tubes and mixed with the ligand in MOPS buffer containing 0.1% (w/v) BSA. Upon addition of a mixture of $G_{\alpha i1}$ (100 nM) and $G_{\beta 1\gamma 2}$ (500 nM), the tubes were incubated on ice for 30 min. The reaction was started by addition of MOPS buffer pH = 7.5 (50 mM), EDTA (1 mM), $MgCl_2$ (3 mM), GDP (4 μM), BSA (0.3% w/v), NaCl (100 mM), DTT (1 mM), and [^{35}S]–GTPγS (5 nM, 1250 Ci/mmol) followed by rapid transfer of the tubes to a water bath at 30 °C. The incubation continued for 45 min. The reaction was terminated by addition of 2 ml of ice-cold stop solution, TNMg (20 mM Tris–HCl pH = 8.0, 100 mM NaCl, and 25 mM $MgCl_2$). The reaction mixture was rapidly filtered through nitrocellulose filters (Millipore, Billerica, MA). Filters were washed four times with 2 ml each of cold TNMg buffer, dried, placed in scintillation vials filled

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